The partial attenuation of Small Hydrophobic (SH) gene deleted RSV is associated with elevated IL-1β responses.

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Abstract

The small hydrophobic (SH) gene of respiratory syncytial virus (RSV), a major cause of infant hospitalisation, encodes a viroporin with of unknown function. SH gene knockout virus (RSV ΔSH) is partially attenuated in vivo but not in vitro, suggesting that the SH protein may have an immunomodulatory role. RSV ΔSH has been tested as a live attenuated vaccine in humans and cattle and here we demonstrate that it protected against viral re-challenge in mice. We compared the immune response to infection with wild type and ΔSH RSV, in vivo using BALB/c mice and in vitro using epithelial cells, neutrophils and macrophages. Strikingly, the IL-1β response to RSV ΔSH infection was greater than wild type RSV, in spite of decreased viral load and when IL-1β was blocked in vivo, viral load returned to wild type levels. A significantly higher IL-1β response to RSV ΔSH was also detected in vitro, with greater magnitude responses in neutrophils and macrophages than epithelial cells. Depleting macrophages (with clodronate liposome) and neutrophils (with anti-Ly6G/ 1A8) demonstrated the contribution of these cells to the IL-1β response in vivo, the first demonstration of neutrophilic IL-1β production in response to viral lung infection. In this study we describe an increased IL-1β response to RSV ΔSH, which may explain the attenuation in vivo and supports targeting the SH gene in live attenuation vaccines.

Importance

There is a pressing need for a vaccine for Respiratory Syncytial Virus (RSV). A number of live attenuated RSV vaccine strains have been developed in which the small hydrophobic (SH) gene has been deleted, even though the function of the SH protein is unknown. The structure of the SH protein has recently been solved showing it is a pore forming protein (viroporin). Here we demonstrate that the IL-1β response to RSV ΔSH is greater in spite of lower viral load, which contributes to the attenuation in vivo. This potentially suggests a novel method by which viruses can evade the host response. As the *Pneumovirinae* all encode similar SH genes this new understanding

45 may also enable the development of live attenuated vaccines for both RSV and other members of
46 the *Pneumovirinae*.

Introduction

Respiratory Syncytial Virus (RSV) is the most significant cause of bronchiolitis and pneumonia in infants for which there is no vaccine (1). Recent advances in the understanding of the infant immune response to vaccination suggest that a live-attenuated vaccine given in infancy may be the most effective approach to prevent RSV infection (2), potentially in combination with maternal immunisation using recombinant F protein (3). This is supported by the successful introduction of the live attenuated influenza vaccine of children (4) in conjunction with immunisation during pregnancy with the trivalent inactivated vaccine (5). One issue with live attenuated RSV vaccines has been balancing immunogenicity and safety (6). Two approaches are used to develop live attenuated vaccines; biological derivation of strains, usually by multiple passage often at lower temperatures, or targeted gene deletion by reverse genetics.

To most effectively attenuate a virus by reverse genetics, understanding the proteins it encodes is required. In the current generation of live attenuated RSV vaccines, genes encoding the non-structural protein 2 (NS2) and the small hydrophobic protein (SH) have been targeted. NS2 acts as an inhibitor of the type I interferon response (7), modulating NF-kappaB (8), but the function of the SH protein is currently unknown. *In silico* analysis suggests that a transmembrane pentamer is the most energetically favourable conformation of SH (9). The formation of SH protein pentamers has been confirmed by cryo-EM studies (10) and SH pentamers enable the passage of ions and small molecules (10-12). When transfected into HEK293 cells, SH is located at the plasma membrane (11) and during RSV infection SH is located to the Golgi (13). These studies suggest that the SH protein belongs to the family of viroporins (14), however the role of the pore encoded by the SH gene is unknown.

Recombinant RSV which does not express the SH protein is partially attenuated in vivo (15) but not in vitro (16). This suggests it may play a role in modulating the immune response with earlier studies showing that RSV SH inhibits TNF signalling (17). In a recent study, SH gene deletion recombinant

bovine RSV (bRSV) is attenuated (18) inducing increased levels of the cytokines IL-1β and TNF. Viral pore proteins have been proposed to modulate the inflammasome, a multi-protein pattern recognition complex that catalyses the cleavage of the pro-forms of IL-1β and IL-18 into their active forms via caspase 1 (19).

We observed that recombinant RSV lacking the SH gene was attenuated and also protective against subsequent virus challenge. We propose that the attenuation observed in vivo following deletion of the RSV SH gene is due to its effect on the host immune response. To test this, we compared the response to infection with wild type RSV (strain A2) and its derivative in which the SH gene has been deleted (RSV ΔSH) in vitro and in vivo. We observed that RSV ΔSH induced significantly greater levels of IL-1β, especially from macrophages and neutrophils. These studies demonstrate increased inflammation to the SH deleted virus, which may contribute to the attenuation.

Materials and Methods

Virus. Biological RSV strain A2 was used as the wild type (WT) and compared to a SH gene deletion recombinant on an A2 background (20). Infectious stocks of virus were grown using the human laryngeal carcinoma cell line, HEp-2. Viral titre was calculated by an immuno-plaque assay using biotinylated goat anti-RSV polyclonal antibody (AbD Serotec, Oxford, UK) to detect plaques. Prior to in vitro and in vivo studies, stocks were screened for LPS contamination. Virus was inactivated by UV irradiation at $1.3x10^5$μJ/cm^2 for 15 min on ice in a CX-2000 Crosslinker (UVP, Cambridge, UK).

Animals. Female BALB/c mice were obtained from Harlan Scientific (Brook House, UK) and used at 6-8 weeks of age. All procedures undertaken were approved by the local ethics review board and performed by personal licensees under the appropriate project license. Experiments were carried out in accordance with Animals (Scientific Procedures) Act 1986. Mice were infected with $2.5x10^5$ PFU virus or media alone in a 100 μl volume intranasally whilst under isoflurane anaesthesia. Animals were weighed prior to RSV challenge and daily thereafter. Where described neutrophils

were depleted with 0.5mg anti-Ly6G (1A8, BioXCell, USA) and IL-1β was blocked with 0.5 mg anti-mIL-1β (B122, BioXCell) in vivo in 500 µl delivered intraperitoneally on d-1 and +1 of primary RSV infection. For macrophage depletion, mice were treated with 100 µl of clodronate liposome (CL) suspension (Boehringer GmbH, Germany), or control empty liposomes (PL), intranasally. After infection bronchoalveolar lavage (BAL) fluids, lung tissues and serum samples were harvested as described previously (21).

RSV viral load. Viral load in vivo was assessed by extracting RNA from frozen lung tissue disrupted in a TissueLyzer (Qiagen, Manchester, UK) using Trizol extraction and then converting it into cDNA. Quantitative RT-PCR was carried out using bulk viral RNA, for the RSV L gene and mRNA using 900 nM forward primer (5'-GAACTCAGTGTAGGTAGAATGTTTGCA-3'), 300 nM reverse primer (5'-TTCAGCTATCATTTTCTCTGCCAAT-3') and 100 nM probe (5'-FAM-TTTGAACCTGTCTGAACAT-TAMRA-3') on a Stratagene Mx3005p (Agilent technologies, Santa Clara, CA). L-specific RNA copy number was determined using a RSV L gene standard.

Cytokine quantification. IL-1β was measured in samples by human or mouse duoset ELISA according to manufacturer's instructions (R & D Systems, Oxford, UK). IL-6 and CXCL1/KC were measured by Luminex (Bio-Rad, UK).

Flow cytometric analysis. Live cells were suspended in Fc block (Anti-CD16/32, BD) in PBS-1% BSA and stained with surface antibodies (Panel 1: RSV M2 82-90 pentamer R-PE (Proimmune, Oxford, UK), CD3-FITC (BD), CD4-APC (BD), CD8-APC Alexa75 (Invitrogen) and CD19-eflour450 (eBioscience); Panel 2: CD11c-FITC (BD), CD80-APC (eBioscience), MHCII-eflour450 (eBioscience), F4/80-PECy7 (eBioscience) and Ly6G-BV605 (BD). For intracellular IL-1β staining, after surface staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD) and stained with IL-1β (BD). Analysis was performed on an LSRFortessa flow cytometer (BD). FMO controls were used for surface stains and an IgG1-PE isotype control was used for IL-1β intracellular staining.

Antigen specific ELISA. A quantitative assay was used to determine serum antibody levels adapted from (22). Plates were coated with 1 µg/ml RSV-antigen and blocked with PBS-1% BSA. Bound IgG was detected with HRP-conjugated goat anti-mouse IgG (AbD Serotec). A dilution series of recombinant murine IgG was used as a standard to quantify RSV specific antibodies. TMB with H_2SO_4 stop was used to detect the response and optical densities read at 450 nm.

In vitro cells. HEp-2 cells were grown in in DMEM (Sigma) with 10% fetal calf serum (FCS), L-glutamine and penicillin/streptomycin. THP-1 cells were grown in RPMI with 10% FCS, L-glutamine penicillin/streptomycin. THP-1 cells were differentiated into phagocyte-type cells by seeding into new tissue culture plates and supplementing with 20 ng/ml PMA and incubating for 24 hours before being rested for a further 24 hours (adapted from (23)). For PBMC, mixed donor pools from three NC24 leucocyte cones were sourced from NHS Blood and Transplant unit in Colindale, UK. RosetteSep CD8 depletion cocktail (StemCell Technologies) was used to prepare CD8 depleted PBMC populations. Cells were dispensed into tissue culture plates at the required density following the addition of 10 U/ml IL-2. Primary neutrophils: Neutrophils were separated from fresh blood collected from healthy donors with written consent according to Local Research Committee guidelines. A modified Percoll method was used (24). Neutrophils were separated on a 70% over 60% Percoll solution, centrifuged at 500xg for 35 min at 22°C, with acceleration/brake $2m/s^2$. This gave a population that was > 90% neutrophils confirmed by differential staining.

In vitro viral infection. Cells were seeded at the following densities (HEp-2 – $5x10^5$, THP-1 – $5x10^5$, PBMC – $5x10^6$, Neutrophils – $5x10^6$) into 24 well plates. Cells were incubated for a minimum of 24 hours at 37°C, 5% CO_2 before infection with ΔSH RSV or WT RSV at MOI 0.5. At time points specified in the results, supernatant was collected and subjected to cytokine analysis by ELISA.

Fluorescence microscopy and imaging. HEp-2 and THP-1 cell lines and primary neutrophils were infected with GFP-tagged RSV (25) 24 or 48 hours prior to imaging at MOI of 0.1. Images were captured using a Nikon Eclipse TE2000 Inverted Microscope, attached to Nikon digital camera (DXM

1200F), with 20x/0.45 magnification. Blue (400-446 nm) fluorescence filters were used to detect GFP.

Statistical Analysis. Calculations as described in figure legends were performed using Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

Results

RSV ΔSH infection is protective against subsequent viral challenge.

SH gene deletion has been proposed as a possible attenuating mutation for the generation of vaccine strains of RSV. To confirm that RSV ΔSH protected against RSV challenge, mice were infected intranasally with either wild type RSV (RSV WT) or RSV ΔSH at $2.5x10^5$ PFU in a 100 µl volume or media alone as a control and then 28 days later challenged intranasally with $2.5x10^5$ PFU RSV WT in 100 µl. Sera was collected prior to challenge infection and RSV WT and ΔSH infection generated an RSV specific IgG response, with the response to ΔSH slightly but not significantly greater than RSV WT (Fig. 1A). Mice that had primary infection with either ΔSH or WT were protected against disease following subsequent RSV challenge, on day 6 and 7 after RSV challenge infection, control treated mice lost significantly more weight ($p<0.001$, Fig. 1B). Interestingly, as we have previously observed (26), there was an acute weight loss on days 1 and 2 in the primed groups. There was no detectable viral load in either primed group, but RSV L gene-specific RNA was detectable in the control group on both days 4 and 7 after RSV infection (Fig. 1C). There was no difference in the IL-1β level in the lung (Fig. 1D) on day 4 after infection, but the control group had significantly more IL-1β in the lungs on day 7. The primed groups recruited more cells to the lungs (Fig. 1E) on day 4 with a greater proportion of $CD4^+$ and $CD8^+$ T cells on day 4. On day 7 after infection, the mice previously infected with RSV WT had significantly more RSV specific CD8 cells (Fig. 1H). Both previously infected groups had significantly higher anti-RSV IgG responses than the control treated mice on day 7 and an increase from the level prior to challenge indicating a memory response to RSV (Fig. 1I).We thus confirm that RSV ΔSH can induce a protective immune response.

RSV lacking the SH gene is attenuated in vivo but induces a greater IL-1β response.

To assess the role of the SH protein during RSV infection, BALB/c mice were infected with $2.5x10^5$ PFU RSV WT or RSV ΔSH (20). Mice infected with RSV WT lost significantly more weight than mice

infected with RSV ΔSH on days 5-7 after infection ($p<0.05$, Fig. 2A). The ΔSH virus was attenuated; markedly less RSV RNA was detected in the RSV ΔSH groups than RSV WT infected animals (Fig. 2B). There was no difference in the total cell count recovered from the lungs (Fig. 2C). However, there was a significantly greater recruitment of $CD4^+$ ($p<0.05$, Fig. 2D), $CD8^+$ ($p<0.05$, Fig. 2E) and $DX5^+$/NK cells ($p<0.05$, Fig. 2F) in the lungs of mice infected with RSV ΔSH on day 2 after infection. Lung IL-6 ($p<0.001$, Fig. 2G) and CXCL1 ($p<0.001$, Fig. 2H) were significantly greater in RSV WT infected mice on day one after infection. IL-1β levels were slightly but not significantly higher in mice infected with RSV ΔSH on days 2 and 4 after infection (Fig. 2I). This was striking because the viral load was so much lower in the RSV ΔSH infected mice, suggesting that the IL-1β response was modulated by the SH gene. IL-1β was blocked during RSV ΔSH infection to determine its contribution to the attenuated phenotype of the RSV ΔSH. Mice were treated with anti-IL-1β on d-1 and d+1 of infection and viral load measured on d4 after infection. RSV ΔSH was significantly attenuated compared to the wild type virus, but anti-IL-1β treatment restored the ΔSH viral load to wild type levels (Fig. 2J).

RSV lacking the SH gene is not attenuated in vitro and induces a greater IL-1β response.

To dissect the effect of SH gene deletion on the IL-1β response, a range of cell types were infected in vitro. HEp-2 cells were infected at an MOI of 0.5 of RSV WT or RSV ΔSH and supernatants collected at various timepoints after infection. RSV ΔSH has been observed to replicate to similar titres as wild type virus in vitro and we observed a similar effect in HEp-2 (Fig. 3A). Levels of IL-1β induced by viral infection of HEp-2 cells were low, but the RSV ΔSH virus induced more IL-1β than RSV WT (Fig. 3B). We have previously observed that macrophages contribute to the inflammatory response to RSV infection (27) and wished to compare the IL-1β response in these cells. THP-1 cells (a macrophage like cell line) and CD8 depleted, adherent PBMC were cultured with RSV WT or RSV ΔSH. RSV WT induced an IL-1β response that was greater than control treated cells, at a level similar to that observed in previous studies (28). RSV ΔSH induced a significantly greater IL-1β response than RSV WT in both THP-1 ($p<0.05$, Fig. 3C) and PBMC ($p<0.05$, Fig. 3D). To determine whether viral

198 replication or viral associated PAMPs were driving the IL-1β response, the IL-1β response was
199 compared in THP cells exposed to live or UV inactivated virus. Inactivation of the virus was
200 confirmed by in vitro plaque assay (data not depicted). Live RSV induced a significantly greater IL-1β
201 response than UV inactivated virus, with both the ΔSH and WT viruses. Since neutrophils are also a
202 significant source of inflammatory cytokines in the lungs after infection, IL-1β levels were measured
203 at 24 hours after RSV infection of primary human neutrophils. Infection of neutrophils with RSV ΔSH
204 led to a significantly greater IL-1β response than RSV WT ($p<0.05$, Fig. 3E). From these studies, we
205 speculate that the SH protein of RSV antagonizes the IL-1β response.

206 IL-1β release following exposure to RSV may be in response to infection of the cell (in cis) or in
207 response extracellular virus or danger signals (in trans). To understand the response in different cell
208 types we determined whether RSV infects these cell types. Cells were cultured with GFP expressing
209 RSV and imaged by fluorescence microscopy. Green fluorescence was detectable in HEp-2 (Fig. 3F)
210 and THP-1 (Fig. 3G) cells after infection with RSV, indicating that there is transcription of virally
211 encoded RNA. More green HEp-2 cells were detectable than THP-1 cells for an equivalent MOI of
212 RSV. Very limited green fluorescence was observed in primary neutrophils at 24 hours after
213 infection, even with a higher MOI of RSV, suggesting they do not support viral replication (Fig. 3H).

214 **IL-1β is produced in response to RSV by macrophages and neutrophils in vivo**

215 Having observed differences in the amount of IL-1β produced by different cell types in vitro, we
216 wished to determine which cell types produce IL-1β in vivo. Cells were isolated from the lungs after
217 RSV WT infection or mock infection with media alone and IL-1β positive cells identified by
218 intracellular cytokine staining in the absence of stimulation. The number of IL-1β^+ Ly6G$^+$ cells
219 (neutrophils, Fig. 4A) was significantly greater in the infected group than the uninfected group.
220 Interestingly the percentage of Ly6G cells positive for IL-1β increased over the course of infection.
221 Detectable expression of IL-1β by CD11c$^+$/ F480$^+$/ MHCII low cells (alveolar macrophages (29), Fig.
222 4B) was highest on days 1 and 2 after infection, but returned to control levels by day 4 after

223 infection. No lymphocytes were observed to be IL-1β positive. A similar profile of IL-1β positive cells
224 was observed following infection with RSV ΔSH.

225 Cell specific depletion was used to determine whether a specific cell type was the major contributor
226 to lung IL-1β after infection. The effect on acute RSV infection (day 1 after infection) of alveolar
227 macrophage depletion (by clodronate liposome (CL) treatment) or neutrophil depletion (using anti
228 Ly6G (1A8) antibody) was compared. 1A8 treatment led to a significant reduction in the number of
229 neutrophils detectable in the lungs (Fig. 4C) and BAL (data not depicted). CL treatment led to a
230 significant decrease in the percentage of $CD11c^{+}/F480^{+}$/ MHCII lo cells (Fig. 4D). Cell depletion had
231 no effect on viral load at day one after infection (Fig. 4E). In spite of the high percentage of IL-1β
232 positive neutrophils detected, neutrophil depletion by 1A8 treatment alone had no effect on IL-1β
233 (Fig. 4F). Interestingly more CXCL1 was detected in the lungs of these mice (Fig. 4G) and there was
234 no effect on IL-6 levels (Fig. 4H). CL treatment alone significantly reduced the level of IL-6 but had no
235 effect on lung levels of IL-1β or CXCL1. Mice that received both clodronate and 1A8 treatment had
236 significantly reduced IL-1β, suggesting that either there is some redundancy in the cells that produce
237 IL-1β or these cells act cooperatively. CL treatment alone without RSV infection did not significantly
238 increase in IL-1β production in the lungs compared to control empty liposome (PL) treatment alone
239 (Fig. 4F). We therefore conclude that IL-1β is produced by a number of different cellular sources,
240 including neutrophils and CL sensitive cells, presumably alveolar macrophages.

Discussion

One rationale for understanding the function of the SH gene is to target it for deletion in live attenuated RSV vaccines. Here we demonstrate enhanced inflammation after infection with RSV lacking the SH gene, particularly in the IL-1β pathway. In the current study we demonstrated that RSV ΔSH protected against viral challenge in the mouse model. A recent study has demonstrated that a bRSV ΔSH vaccine is highly protective in infant calves, even in the presence of maternal antibody (30) and a live attenuated RSV ΔSH vaccine, with additional point mutations, (Medi-559) has been shown to be safe and immunogenic in clinical trials in children (31). Data from chimpanzee studies (32), which showed moderate attenuation, suggest SH alone would be insufficient for a safe live vaccine, though it has been shown to be highly effective in cattle (30). Whilst addition of ΔSH to other temperature sensitive vaccines had a marginal effect (33, 34) on attenuation, we speculate that SH has an immunomodulatory function and its deletion as part of a live attenuated RSV vaccine design would be beneficial.

The observation that deletion of RSV SH leads to an increase in IL-1β is supported by studies in bRSV, where a ΔSH vaccine strain induced higher levels of IL-1β in the lungs of infected cattle (18). In the current study, blockade of IL-1β prior to infection increased viral load, supporting the idea that SH might enable immunomodulation. Interestingly, the bRSV ΔSH also induced greater levels of apoptosis, which can be associated with inflammasome activation. In contrast, a study with different live attenuated RSV vaccines showed that the most heavily attenuated vaccine (rA2cp248/404/1030/ΔSH) induced lower responses in nasal wash samples to a number of cytokines compared to other live attenuated vaccines (6). However, the virus tested is highly attenuated, and the reduced cytokine response is likely a consequence of a strong attenuation of replication related to the point mutations in the backbone of the virus rather than the SH deletion. A recent study by Triantafilou et al proposed that the RSV SH protein activates the inflammasome (35). Unlike the current study, the virus used by Triantafilou et al has the SH gene replaced with GFP. We believe that

266 there are flaws in the published study by Triantafillou et al that warrant further investigation: figures
267 showing the relative growth of the two viruses are not included in the study, the study is only
268 performed at a single time point in a single cell line using a high MOI of virus and there is no
269 statistical analysis comparing the response between the viruses. In contrast to the published study
270 we have carefully dissected the inflammasome response to RSV infection, with and without the SH
271 protein both in vivo and in vitro performing multiple time course studies using primary cells, cell
272 lines and a mouse model.

273 We observed that both neutrophils and clodronate sensitive alveolar macrophages contribute to the
274 lung IL-1β response (in vivo and in vitro). We propose a model where the alveolar macrophages act
275 as sentinels for infection initiating the immune response and the neutrophils then amplify the signal.
276 Whilst the role of alveolar macrophages in initiating the immune response to RSV infection by
277 cytokine production has been explored by us (27, 36) and other groups (37), the role of neutrophils
278 in RSV infection is not known. Studies have shown IL-9 (38), TNF (39) and chemokine production (40)
279 by neutrophils in response to RSV. Neutrophils are a source of IL-1β in lung injury models (41), but
280 this is the first study that has demonstrated IL-1β production by neutrophils in response to
281 respiratory viral infection. Airway neutrophilia is observed in patients with bronchiolitis (42) and an
282 increase in neutrophils is detected in blood prior to the influx of CD8 T cells (43). We have previously
283 observed a persistent airway neutrophilia following regulatory T cell depletion (44) suggesting they
284 are associated with disease, but other studies have demonstrated neutrophils can have a protective
285 (45), anti-viral role (46). Whilst RSV RNA and protein has been observed in airway neutrophils from
286 infected infants (47) the data generated using GFP expressing virus suggests that macrophages but
287 not neutrophils can support viral replication.

288 We speculate that SH is an immune evasion protein and that this mechanism of immune evasion
289 may be conserved across other viruses, SH genes are found in all of the subfamily *Pneumovirinae*
290 and some of the *Paramyxovirinae* including Mumps (48) and human parainfluenza virus 5 (hPIV5)

(49). The SH proteins from human metapneumovirus (hMPV) (50), bRSV (51), hPIV5 (49) and Mumps (52) are non-essential for in vitro growth and immunomodulatory function has been described for the SH proteins from PIV5 (49), bRSV (18) and hMPV (53), which also encodes a viroporin (54). We hypothesize that the pore structure of the SH protein (11) facilitates this immunomodulatory function, counteracting the activation of NLRP3 by ionic changes induced by RSV infection (28). However not all viroporins inhibit inflammasome function, for example the M2 protein of influenza A encodes a viroporin, which has been demonstrated to activate the inflammasome (55). The differences between RSV SH and influenza M2 may be due to the differences in structure (14). Of note, another viroporin with a similar structure to RSV SH, influenza B NB, has a similar, limited effect on the replication of influenza in vitro (56). Alternatively, SH has been shown to form a complex with the G protein (57), which has also been shown to have an immunomodulatory function (58) and potentially the complex is important, more research is required to determine the exact mechanism of action. In addition to depletion in live vaccines, targeting the SH protein may be open up novel anti-viral therapies and a recent study has demonstrated that its function as an ion pore can be blocked with a small-molecule inhibitor, pyronin B (12). In the current study, we show that deletion of the SH gene changes the immune response on infection, increasing IL-1β responses by an as yet undetermined mechanism, and believe that further research on this aspect of SH gene function is important in the development of an RSV vaccine.

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494 **Figure Legends**

495 **Figure 1. RSV ΔSH is protective against RSV infection.** Mice were infected intranasally with $2.5x10^5$
496 PFU either RSV WT (grey bars/ diamonds), RSV ΔSH (black bars/ triangles) or control treated (white
497 bars/ circles), 4 weeks later all mice were challenged with RSV WT. Anti-RSV IgG was measured by
498 ELISA one day before RSV challenge (A). Weight change (B), lung viral load (C), lung IL-1B (D) and
499 lung cell number (E) were measured after infection. Lung $CD4^+$ (F) and $CD8^+$ (G) T cells on day 4 and
500 day 7 and lung RSV specific CD8 T cells on day 7 were measured by flow cytometry (H). Anti-RSV IgG
501 was measured on day 7 after infection by ELISA (I). Points represent individual animals (A,I). Points/
502 bars represent mean +/- SEM of n=5 animals (B-H) *p<0.05, **p<0.01, ***p<0.001, measured by
503 multiple t tests and Holm-Sidak correction (B) or ANOVA (A,C-I).

504 **Figure 2**. **Recombinant RSV lacking the SH gene (RSV ΔSH) is attenuated in vivo but induces a**
505 **greater IL-1β response than wild type.** Mice were infected intranasally with RSV WT or RSV ΔSH.
506 Weight loss (A), lung viral load (B) and lung cell number (C) were measured after infection. Lung
507 $CD4^+$ T (D), $CD8^+$ T (E) and $DX5^+$ NK (G) cells were measured by flow cytometry. IL-6 (G), CXCL1 (H)
508 and IL-1β (I) were measured in lung supernatants by ELISA. Mice were treated with anti IL-1β prior to
509 infection with RSV ΔSH and viral load measured on d4 after infection (J). Points represent mean of
510 n>5 mice +/- SEM, *p<0.05, **p<0.01, calculated by multiple t tests and Holm-Sidak correction.

511 **Figure 3**. **Recombinant RSV lacking the SH gene induces a greater IL-1β response than wild type *in***
512 ***vitro*.** HEp-2 cells were infected with 0.5 MOI RSV WT or RSV ΔSH, viral load was assessed by plaque
513 assay (A). Supernatants were collected and analysed for IL-1β level by ELISA following infection of
514 HEp2 cells (B), THP-1 cells (C), PBMC (D), and neutrophils (E). HEp 2 (F), and THP-1 (G) were infected
515 with MOI 0.1 RSV GFP and primary neutrophils (H) were infected with MOI 0.25 RSV and imaged at
516 24 hours after infection. Points represent mean +/- SEM of n=3 repeats of HEp2, PBMC and THP-1
517 cells and 3 individual PBMC and neutrophil donors.

518 *p<0.05, ***p<0.001 between ΔSH and wt RSV, # p<0.05 between ΔSH and UV ΔSH , calculated by
519 two way ANOVA (A-D) or ANOVA (E). Images representative of 2 studies.

520

521 **Figure 4. IL-1β is produced by neutrophils and macrophages in vivo.** Mice were infected with RSV
522 WT (grey diamonds) or control treated (white circles) intranasally. Expression of IL-1β by $Ly6G^{+}$
523 (neutrophils: A) and $CD11c^{+}/F480^{+}$/MHCII lo (alveolar macrophages: B) was measured by flow
524 cytometry at various time points after infection. Mice were treated with anti-Ly6G depleting
525 antibody (1A8) or control antibody (Con) intraperitoneally, and clodronate liposomes (CL) or empty
526 liposomes (PL) intranasally prior to RSV WT infection. Neutrophil (C) and alveolar macrophage (D)
527 numbers were analysed by flow cytometry, RSV L gene (E) by RT-PCR and lung IL-1β (F), CXCL1 (G)
528 and IL-6 (H) were measured on day 1 after infection. Points represent mean +/- SEM of n= 5 mice
529 (A,B) and individual animals, bars represent mean, n=5 (C-H). *p<0.05, **p<0.01, ***p<0.001,
530 calculated by multiple t tests and Holm-Sidak correction (A-B) or ANOVA (C-H).

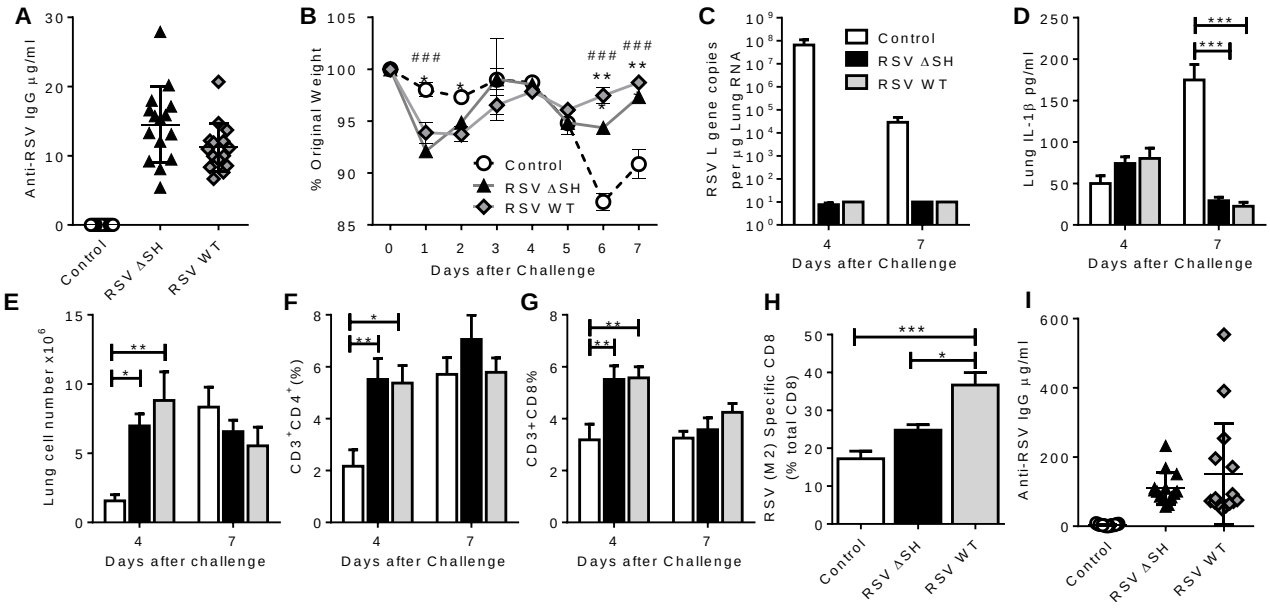


Figure 1. RSV ΔSH is protective against RSV infection. Mice were infected intranasally with 2.5x10^5 PFU either RSV WT (grey bars/ diamonds), RSV ΔSH (black bars/ triangles) or control treated (white bars/ circles), 4 weeks later all mice were challenged with RSV WT. Anti-RSV IgG was measured by ELISA one day before RSV challenge (A). Weight change (B), lung viral load (C), lung IL-1B (D) and lung cell number (E) were measured after infection. Lung $CD4^+$ (F) and $CD8^+$ (G) T cells on day 4 and day 7 and lung RSV specific CD8 T cells on day 7 were measured by flow cytometry (H). Anti-RSV IgG was measured on day 7 after infection by ELISA (I).Points represent individual animals (A,I). Points/ bars represent mean +/- SEM of n=5 animals (B-H). *p<0.05, **p<0.01, ***p<0.001, measured by multiple t tests and Holm-Sidak correction (B) or ANOVA (A,C-I).

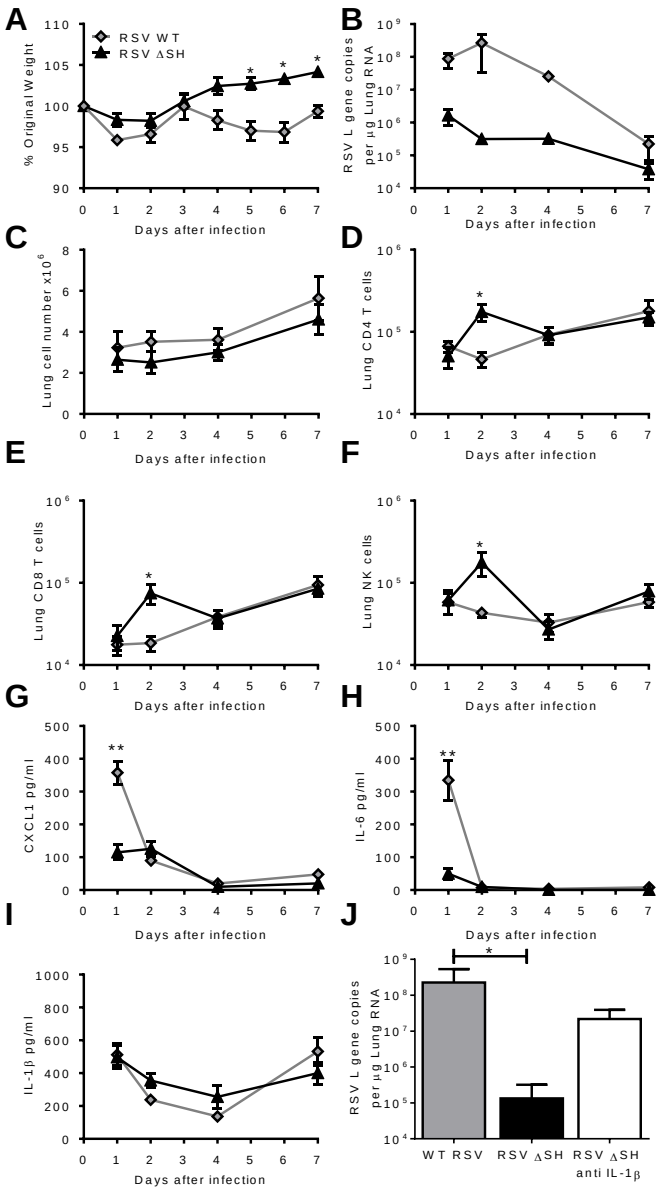


Figure 2. **Recombinant RSV lacking the SH gene (RSV ΔSH) is attenuated in vivo but induces a greater IL-1β response than wild type.** Mice were infected intranasally with RSV WT or RSV ΔSH. Weight loss (A), lung viral load (B) and lung cell number (C) were measured after infection. Lung $CD4^+$ T (D), $CD8^+$ T (E) and $DX5^+$ NK (G) cells were measured by flow cytometry. IL-6 (G), CXCL1 (H) and IL-1β (I) were measured in lung supernatants by ELISA. Mice were treated with anti IL-1β prior to infection with RSV ΔSH and viral load measured on d4 after infection (J). Points represent mean of n>5 mice +/- SEM, *p<0.05, **p<0.01, calculated by multiple t tests and Holm-Sidak correction (A-I and Kruskal-Wallis (J).

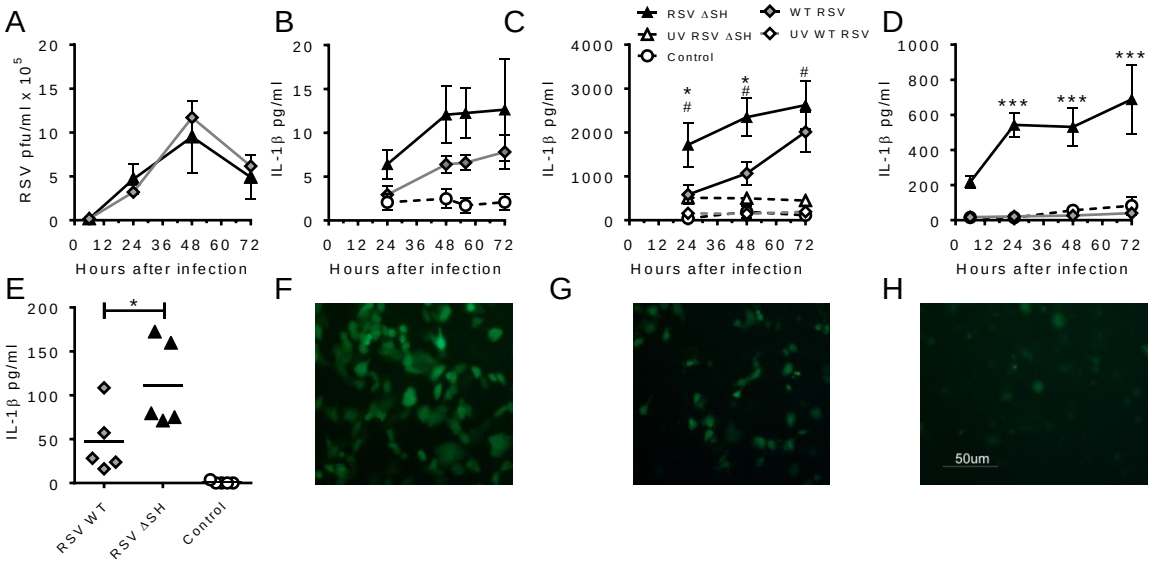


Figure 3. **Recombinant RSV lacking the SH gene induces a greater IL-1β response than wild type in vitro.** HEp-2 cells were infected with 0.5 MOI RSV WT or RSV ΔSH, viral load was assessed by plaque assay (A). Supernatants were collected and analysed for IL-1β level by ELISA following infection of HEp2 cells (B), THP-1 cells (C), PBMC (D), and neutrophils (E). HEp 2 (F), and THP-1 (G) were infected with MOI 0.1 RSV GFP and primary neutrophils (H) were infected with MOI 0.25 RSV and imaged at 24 hours after infection. Points represent mean +/- SEM of n=3 repeats of HEp2, PBMC and THP-1 cells and 3 individual PBMC and neutrophil donors. *p<0.05, ***p<0.001 between ΔSH and wt RSV, # p<0.05 between ΔSH and UV ΔSH , calculated by two way ANOVA (A-D) or ANOVA (E). Images representative of 2 studies.

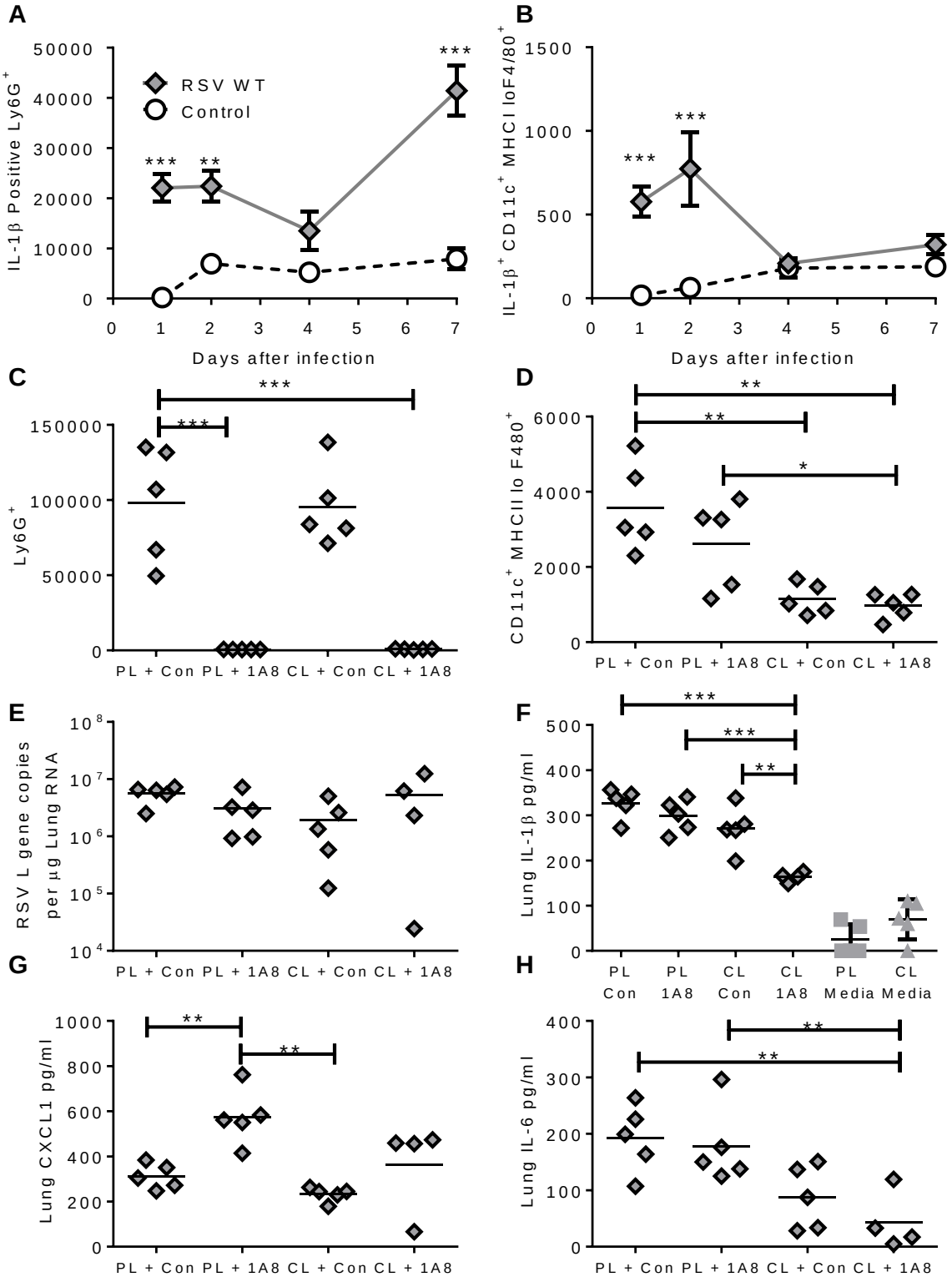


Figure 4. IL-1β is produced by neutrophils and macrophages in vivo. Mice were infected with RSV WT (grey diamonds) or control treated (white circles) intranasally. Expression of IL-1β by Ly6G$^+$ (neutrophils: A) and CD11c$^+$/F480$^+$/MHCII lo (alveolar macrophages: B) was measured by flow cytometry at various time points after infection. Mice were treated with anti-Ly6G depleting antibody (1A8) or control antibody (Con) intraperitoneally, and clodronate liposomes (CL) or empty liposomes (PL) intranasally prior to RSV WT infection. Neutrophil (C) and alveolar macrophage (D) numbers were analysed by flow cytometry, RSV L gene (E) by RT-PCR and lung IL-1β (F), CXCL1 (G) and IL-6 (H) were measured on day 1 after infection. Points represent mean +/- SEM of n= 5 mice (A,B) and individual animals, bars represent mean, n=5 (C-H). *p<0.05, **p<0.01, **p<0.001, calculated by multiple t tests and Holm-Sidak correction (A-B) or ANOVA (C-H).